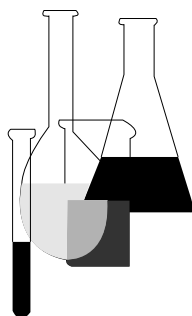




# Ecological Effects Test Guidelines

## OPPTS 850.6200 Earthworm Subchronic Toxicity Test



**“Public Draft”**

## INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations.

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD).

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U. S. Environmental Protection Agency under the Toxic Substances Control Act (15 U.S.C. 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U.S.C. 136, *et seq.*).

**Public Draft Access Information:** This draft guideline is part of a series of related harmonized guidelines that need to be considered as a unit. *For copies:* These guidelines are available electronically from the EPA Public Access Gopher (gopher.epa.gov) under the heading “Environmental Test Methods and Guidelines” or in paper by contacting the OPP Public Docket at (703) 305-5805 or by e-mail: guidelines@epamail.epa.gov.

**To Submit Comments:** Interested persons are invited to submit comments. By mail: Public Docket and Freedom of Information Section, Office of Pesticide Programs, Field Operations Division (7506C), Environmental Protection Agency, 401 M St. SW., Washington, DC 20460. In person: bring to: Rm. 1132, Crystal Mall #2, 1921 Jefferson Davis Highway, Arlington, VA. Comments may also be submitted electronically by sending electronic mail (e-mail) to: guidelines@epamail.epa.gov.

**Final Guideline Release:** This guideline is available from the U.S. Government Printing Office, Washington, DC 20402 on *The Federal Bulletin Board*. By modem dial 202-512-1387, telnet and ftp: fedbbs.access.gpo.gov (IP 162.140.64.19), or call 202-512-0135 for disks or paper copies. This guideline is also available electronically in ASCII and PDF (portable document format) from the EPA Public Access Gopher (gopher.epa.gov) under the heading “Environmental Test Methods and Guidelines.”

## **OPPTS 850.6200 Earthworm subchronic toxicity test.**

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136, *et seq.*) and the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601).

(2) **Background.** The source materials used in developing this harmonized OPPTS test guideline is the OPPT guideline under 40 CFR 795.150 Earthworm Toxicity Test (proposed in the FEDERAL REGISTER of June 25, 1991 (56 FR 29155)).

(b) **Purpose.** This guideline is intended for use in developing data on the toxicity of chemical substances and mixtures (“chemicals”) subject to environmental effects test regulations under TSCA. The guideline sets forth the procedures and conditions for conducting this toxicity test. The EPA ) will use data from this test in assessing the hazard of a chemical to earthworms in the soil environment.

(c) **Definitions.** The definitions in section 3 of TSCA and the definitions in 40 CFR Part 792—Good Laboratory Practice Standards (GLPS) apply to this guideline. The following definitions also apply:

*Artificial soil* means a defined dry weight mixture of 68 percent of No. 70 mesh silica sand, 20 percent kaolin clay, 10 percent sphagnum peat moss, and 2 percent calcium carbonate. These ingredients are weighed and mixed in the above proportions and moistened to 35 percent (by weight) with deionized/distilled water.

*Behavioral symptoms* are indicators of toxicity to earthworms such that a distinct difference in position in the test container can be identified, e.g., below surface or on the surface; writhing on the surface; stiffened and shortened on the surface or elongated and pulsing; or inactive below surface in a ball.

*Clitellum* means a glandular portion of the anterior epidermis, appearing as saddle-shaped or annular, usually differentiated externally by color.

*Culture* means the animals which are raised on-site or maintained under controlled conditions to produce test organisms through reproduction.

*EC50* means that test substance concentration calculated from experimentally-derived growth or sublethal effects data that has affected 50 percent of a test population during continuous exposure over a specified period of time.

*LC50* means that experimentally derived concentration of test substance that is estimated to kill 50 percent of a test population during continuous exposure over a specified period of time.

*Lowest observed effect concentration (LOEC)* means the lowest treatment (i.e., test concentration) of a test substance that is statistically different in adverse effect on a specific population of test organisms from that observed in controls.

*Mature or adult worms* means a condition of the worm exhibiting a clitellum in the anterior of the body.

*Mortality* means the lack of movement by the test organism in response to a definite tactile stimulus to the anterior end. Also, because earthworms tend to disintegrate rapidly after death, the absence of organisms in the enclosed soil test container is considered to mean death has occurred.

*No observed effect concentration (NOEC)* means the highest treatment (i.e., test concentration) of a test substance that shows no statistical difference in adverse effect on a specific population of test organisms from that observed in controls.

*Pathological symptoms* means toxic effects, such as surface lesions and midsegmental swellings or general ulcerated areas on the surface of the earthworm.

*Test mixture* means the test substance/artificial soil mixtures which the earthworms are exposed to during the test.

*Test substance* means any compound used in artificial soils spiked for laboratory testing of toxicity.

**(d) Test procedures—(1) Summary of the test.** (i) Test chambers are filled with appropriate amounts of test mixtures.

(ii) This toxicity test may be done by placing earthworms in test chambers containing test mixtures and allowing earthworms to ingest this test mixture soil ad libitum.

(iii) Acclimated earthworms are introduced into the test and control chambers by stratified random assignment.

(iv) Earthworms in the test and control chambers should be observed every 7 days and the findings should be recorded and dead earthworms removed.

(v) The pH, temperature, and the concentration of the test mixtures should be measured at 7 day intervals in each test chamber.

(vi) Initial weight of earthworm should be between 300 to 600 g per container.

(vii) Concentration-response curves, LC50, EC50, LOEC, NOEC values, and 95 percent confidence intervals for the test substance are developed from the data collected during the test.

(2) **Range-finding test.** (i) If the toxicity of the test substance is not already known, a range-finding test should be performed to determine the range of concentrations to be used in the definitive test.

(ii) The earthworms should be exposed (for at least 28 days) to a range of concentrations of the test substance (e.g., 0.1, 1.0, 10, 100, 1,000 mg/kg dry weight artificial soil).

(iii) Nominal concentrations are acceptable and no replication is required. If the LC50 value is >1,000 mg test substance (100 percent active ingredient) per kilogram dry weight of artificial soil, the definitive test does not have to be done.

(3) **Definitive test.** (i) This test is designed to determine a concentration-mortality curve at 28 days and estimate the respective LC50, EC50, LOEC, NOEC values and 95 percent confidence intervals.

(ii) If data permit, the concentration-response curves, LC50, EC50, LOEC, NOEC values, and 95 percent confidence interval also should be determined for 7, 14, and 21 days.

(iii) This toxicity test uses earthworms which are maintained in direct contact with an artificial soil allowing earthworms to ingest contaminated soil *ad libitum*.

(iv) A minimum of 30 earthworms exposed to each of 5 or more test concentrations and a control should be tested.

(v) Test concentrations should be chosen in a geometric series in which the ratio is between 1.5 and 2.0 mg/kg (e.g., 2, 4, 8, 16, 32, and 64 mg/kg). All test concentrations should be based on milligram of test chemical (100 percent active ingredient) per kilogram of artificial soil (air-dry weight).

(vi) Ten earthworms per container of 200 g (dry weight) artificial soil should be placed in three replicates for each concentration and control. The distribution of individual earthworms among the test chambers should be randomized. Test concentrations in artificial soil should be analyzed for test chemical concentrations prior to the start of the test and at days 7, 14, 21, and 28 as minimum.

(vii) The living earthworms should be placed on the surface of the medium and the jar capped and secured without making an airtight seal.

(viii) Any changes in soil temperature should not exceed 3°C per day or 1°C per hour. Earthworms should be held for a minimum of 7 days at the test temperature prior to testing.

(ix) Every test should include a negative control consisting of uncontaminated artificial soil, conditions, procedures, and earthworms from the same group used in the definitive test as shown, except that none of the test substance is added.

(x) The test duration is 28 days.

(4) **Test results.** (i) Death is the primary criterion used in this test guideline to evaluate the toxicity of the test substance.

(ii) In addition to death, weight loss, behavioral symptoms and pathological symptoms should be recorded.

(iii) Each test and control chamber should be checked for dead or affected earthworms and observations recorded 7, 14, 21, and 28 days after the beginning of the test or within 1 hour of the designated times. Missing earthworms should be considered to have died.

(iv) Mortality is assessed by emptying the test medium on a glass or other inert surface, sorting earthworms from the test mixture and testing their reaction to a gentle mechanical stimulus. Any adverse effects (e.g., weight loss, behavioral or pathological symptoms) are noted and should be reported. The medium is returned to each container.

(v) The 28-day test result is be unacceptable if:

(A) More than 20 percent of control organisms die; or

(B) The total mean weight of the earthworms in the control containers declines significantly during the test (i.e., by 30 percent).

(vi) Mortality is checked and recorded at days 7, 14, 21, and 28.

(vii) The mortality data should be used to calculate LC50 values and their 95 percent confidence limits, and to plot concentration-response curves at days 7, 14, 21, and 28.

(viii) The sublethal effects and growth (i.e., fresh weight) data should be used to plot concentration-response curves, calculate EC50 values, and determine LOEC and NOEC values. Appropriate statistical methods (e.g., one-way analysis of variance and multiple comparison test) should be used to test for significant differences between treatment means and determine LOEC and NOEC.

(5) Analytical measurements—(i) Artificial soil analysis. During the test, the temperature and pH should be measured in the artificial soil at the beginning of the test(zero-hour), and every 7 days thereafter.

(ii) Measurement of test substance.

(A) The concentration of test substance in artificial soil should be measured at a minimum in each test chamber at the beginning (zero-hour, before earthworms are added) and every 7 days thereafter.

(B) The analytical methods used to measure the amount of test substance in a sample should be validated before beginning the test. The accuracy of a method should be verified by a method such as using known additions. This involves adding a known amount of the test substance to three samples of artificial soil taken from the test chamber and the same number of earthworms as are used in the test. The measured concentration of the test substance in those samples should span the concentration range to be used in the test. Validation of the analytical method should be performed on at least two separate days prior to starting the test.

(C) An analytical method is not acceptable if likely degradation products of the test substance give positive or negative interferences, unless it is shown that such degradation products are not present in the test chambers during the test.

(D) In addition to analyzing samples of artificial soil, at least one reagent blank, containing all reagents used, should also be analyzed.

(E) The measured concentration of the test substance in artificial soil in any chamber during the test should not vary more than 50 percent from the measured concentration prior to initiation of the test; concentration measurements should be as described by Neuhauser et al., in paragraphs (g)(5) and (g)(6) of this guideline, or an equivalent method.

(F) The mean measured concentration of test substance in artificial soil (dry weight) should be used to plot all concentration-response curves and to calculate all LC50, EC50, LOEC, and NOEC values.

(G) The total carbon (TC) should be determined as measured by the method of Plumb described in paragraph (g)(7) of this guideline, or an equivalent method.

(iii) **Numerical.** The statistical methods recommended for use in calculating the LC50 and EC50 values include probit, logit, moving average, and binomial.

(e) **Test conditions**—(1) **Test species**—(i) **Selection.** The test species for this test is the earthworm *Eisenia fetida andrei* (Bouche). The species identity of the test organism should be verified using appropriate taxonomic keys as described by Fender in paragraph (g)(2) of this guideline, or an equivalent method.

(ii) **Age and condition of earthworms.** (A) Adult earthworms, 300–600 mg, are to be used to start the test.

(B) Earthworms used in toxicity tests should be purchased from a commercial source that can verify the species. Once verified, cultures should be maintained at the test facility. Records should be kept regarding the source of the initial stock and culturing techniques. All organisms used for a particular test should have originated from the same population (culture).

(C) All newly acquired earthworms should be quarantined and observed for at least 14 days prior to use in a test.

(D) Earthworms should not be used if they have been under stress from too much or a lack of moisture as described by Reinecke and Venter in paragraph (g)(8) of this guideline, or an equivalent method; excessive or inadequate food or temperature as described by Tomlin and Miller in paragraph (g)(11) of this guideline, or an equivalent method; pH variation as described by Satchell and Dottie in paragraph (g)(9) of this guideline, or an equivalent method; or crowding. Any of these conditions will produce earthworms that may not be healthy.

(iii) **Preparation.** Sufficient numbers of earthworms should be harvested and sorted to insure that healthy individuals are used for the test. Any animals that appear to be injured should not be used in the test and must be discarded.

(iv) **Acclimation of test earthworms.** Adult earthworms should be handled with care. Earthworms should be held for a minimum of 7 days in uncontaminated soil at the test temperature prior to testing.

(v) **Feeding.** (A) Substrate food for culturing *Eisenia fetida andrei* should be saturated (water) alfalfa (*Medicago sativa*) pellets.

(B) The earthworms are not fed during the test period.

(2) **Facilities**—(i) **General.** Facilities needed to perform this test include:

(A) Apparatus for providing continuous lighting.

(B) Chambers for exposing test earthworms to the test substance.

(C) A mechanism for controlling and maintaining the artificial soil temperature and relative humidity during the holding, acclimation, and test periods.

(ii) **Construction materials.** (A) Construction materials and equipment that contact test mixtures should not contain substances that can be leached or dissolved into artificial soil in quantities that can affect the test results. Material and equipment that contact test mixtures should be chosen to minimize sorption of test substances. Hard glass jars are pref-



erable and should be heated in an ashing oven between tests; soft glass jars should be used only once.

(B) Polyethylene containers (rectangular dish pans measuring  $32.5 \times 27.5 \times 12.5$  cm) for culturing earthworms, a mechanism (e.g., environmental chamber) for maintaining temperature and relative humidity of the cultures during culturing, and separate facilities for testing are required.

(C) Testing containers (e.g. 1 pt glass canning jars) and lids, and suitable balances to measure soil mixtures and sample weights should also be used.

(D) Relative humidity should be maintained above 85 percent. An open pan of water can be used for this purpose to prevent moisture loss from the containers.

(iii) **Test chambers.** (A) Glass canning jars, 1-pt capacity, or their equivalent, should be used for testing.

(B) The lids should be reversed (i.e., turned upside down), loosely capped and secured without making an airtight seal to reduce evaporation and permit air exchange.

(iv) **Cleaning of test system.** The test chambers should be cleaned before each test following standard laboratory procedures. If soft glass is to be used it must only be used once and then thrown away.

(v) **Medium preparation.** (A) For each concentration tested and controls, enough artificial soil must be prepared by recipe to yield 270 g of artificial soil (wet weight) per replicate. A dry weight mixture of 68 percent of No. 70 mesh silica sand, 20 percent kaolin clay, and 10 percent sphagnum peat moss are mixed until evenly distributed.

(B) Up to 2 percent pulverized calcium carbonate may be added to adjust the soil pH to  $6.5 \pm 0.5$ .

(C) An appropriate amount of high purity water (e.g, 70 g per 200 g of dry soil) is added to the artificial soil and mixed with the artificial soil to raise the artificial soil moisture level to 35 percent by weight to yield a total weight of 810 g artificial soil at 35 percent moisture.

(D) Appropriate portions of the artificial soil are mixed thoroughly with appropriate amounts of test substance to yield three replicates for each test concentration. Each test mixture is divided into three equal quantities of about 270 g as determined by weight. Each portion is placed into a separate 1-pt jar and represents one replicate for exposing 10 earthworms at the same concentration. Three replicates for negative and, if necessary, solvent controls are prepared from untreated portions of the artificial soil mixture.

(E) If a solvent is used, the opened chambers are placed in a hood for 24 hours to evaporate the solvent prior to adding the earthworms.

(F) Prior to the addition of earthworms, a 10-g sample should be removed from each replicate to measure pH and test concentrations.

(3) **Test parameters**—(i) **Loading.** The number of earthworms placed in a test chamber should not be so great as to affect the results of the test. The weight of the individual earthworms should be between 300 mg and 600 mg each. The earthworms are selected from the culture randomly into groups of 10. These groups are then randomly assigned to the test containers and then weighed such that they do not differ more than + 10 percent among the replicates.

(ii) **Temperature.** (A) The test soil temperature should be  $22 \pm 2^{\circ}\text{C}$  as described by Edwards in paragraph (g)(1) of this guideline, or using an equivalent method.

(B) Temperature should be measured and reported at the beginning of the test and on days 7, 14, 21, and 28. The temperature should be measured at least hourly in one test container.

(iii) **Light.** (A) Replicates should be illuminated continuously with incandescent or fluorescent lights as described by Edwards in paragraph (g)(1) of this guideline, or using an equivalent method.

(B) Light intensity should be about 400 lx measured at the artificial soil surface.

(C) Light intensity should be measured at least once during the test at the surface of the container and checked weekly in the test chambers.

(f) **Reporting.** (1) The sponsor should submit all data developed by the test that are suggestive or predictive of toxicity and all concomitant gross toxicological manifestations. The reporting of test data should include the following information:

(i) Test Background including the name of the sponsor, testing laboratory, principal investigator, and dates of testing.

(ii) A detailed description of the test chemical including its chemical identification (CAS Registry No., trade name, common name), source, lot number, composition (identity and concentration or major ingredients and major impurities), known physical and chemical properties, empirical formula, water solubility, vapor pressure, manufacturer, method of application, and any carriers or other additives used and their concentrations. The volume or mass of any carriers should be reported. An exact description of how the test substance has been mixed into the artificial soil.

(iii) Detailed information about the earthworms used as brood stock, including the scientific name and method of verification, age, source, treatments, feeding history, and culture method.

(iv) A description of the test situation, especially if there was a deviation from this test guideline as described above in soil preparation (paragraph (e)(2)(v)(A) of this guideline), addition of the chemical, culturing of the test species, lighting, pH, temperature, replicates, or the number of organisms per container.

(v) A description of the test container used, its size, volume and weight of soil used in each container, number of test organisms per container, number of test containers per concentration, conditioning of the test container, description of the method of test chemical introduction into the test medium (e.g., as a powder), stock solution used or not, and time between mixing of the stock solution and introduction of the earthworms.

(vi) The concentration in artificial soil at the beginning of the test and the actual concentrations of the test chemical (if measured) in the soil before (day 0), during (day 7, 14, 21) and upon the conclusion of the test (day 28) and the dates the analyses were performed.

(vii) The total organic carbon (TOC) of the soil mixture.

(2) The reported results should include:

(i) The number and percentage of organisms that were killed or showed any adverse effects at each test concentration, including controls, in each test jar at each observation period.

(ii) Concentration response curves fitted to mortality data at 7, 14, 21, and 28-day periods. A statistical test of goodness-of-fit should be performed and reported.

(iii) The LC50/EC50 values and the 95 percent confidence limits using the mean measured test concentration and the methods used to calculate both the LC50/EC50; also the LOEC and NOEC values and the confidence intervals by the Trimmed Spearman- Karber method as described by Hamilton et al., in paragraph (g)(3) of this guideline, or an equivalent method. The probit technique should follow the methods described by Weber et al., in paragraph (g)(12) of this guideline, or an equivalent method. Appropriate statistical methods (e.g., one-way analysis of variance and multiple comparison test) should be used to test for significant differences between treatment and determine the LOEC and NOEC.

(iv) All chemical analyses of test material including methods, method validations, and reagent blanks.

(v) The data records for the culture and lighting.

- (vi) Moisture content for the test mixture at start of test.
- (vii) The pH and temperature values at start of test and on days 7, 14, 21, and 28 of the test.
- (viii) Any deviation from this test guideline and anything unusual about the test (e.g., equipment failure, fluctuations in temperature, pH, or other environmental conditions).
- (g) References. For additional background information on this test guideline the following references should be consulted:
- (1) Edwards, C.A. *Report of the second stage in development of a standardized laboratory method for assessing the toxicity of chemical substances to earthworms*. The Artificial Soil Test. DG X1/AL/82/43, Revision 4 (1984).
  - (2) Fender, W.M. *Earthworms of the Western United States*. Part 1. Lumbricidae, *Megadrilologica*, 4: 93–129 (1985).
  - (3) Hamilton, M.A. et al. Trimmed Spearman-Kärber method for estimating median lethal concentrations in toxicity bioassays. *Environmental Science and Toxicology*; 11(7):714–717 (1977). Correction: Ibid 12: 417 (1978).
  - (4) Hartenstein, R. et al. Reproductive potential of the earthworm *Eisenia foetida*., 43: *Oecologia*, 329–340 (1979).
  - (5) Neuhauser, E.F. et al. Contact and artificial soil tests using earthworms to evaluate the impact of wastes in soils, In: Hazardous and Industrial Soil Waste Testing: Fourth Symposium, ASTM STP 886. J.K. Petros, Jr. and R.A. Conway, eds., (American Society for Testing and Materials. Philadelphia, PA 1986) pp. 192–203.
  - (6) Neuhauser, E.F. et al. The toxicity of selected organic chemicals to the earthworm *Eisenia fetida*. *Journal of Environmental Quality*, 14: 383–388 (1985).
  - (7) Plumb, R.H., Jr. Procedures for handling and chemical analysis of sediment and water samples. Technical Report EPA/CE-81-1, prepared by Great Lakes Laboratory, State University College at Buffalo, Buffalo, NY., for the U.S. Environmental Protection Agency/Corp. of Engineers Technical Committee on Criteria for Dredged and Fill Material. U.S. Army Engineer Waterways Experiment Station, CE, Vicksburg, MS. (1981).
  - (8) Reinecke, A.J. and Venter, J.M. Moisture preferences, growth and reproduction of the compost worm *Eisenia fetida* (Oligochaeta), *Biology of Fertility Soils*, 3: 135–141 (1987).

(9) Satchell, J.E. and Dottie, D.J. Factors affecting the longevity of earthworms stored in peat. *Journal of Applied Ecology*, 21: 285–291 (1984).

(10) Stafford, E.A. and Edwards, C.A. Comparison of heavy metal uptake by *Eisenia faetida* with that of other common earthworms. Final Technical Report. Entomology Department, Rothamsted Experiment Station, Harpenden, Herts. AL5 2JQ, U.K. U.S. Army Contract DAJA 45–84–0027 (1985).

(11) Tomlin, A.D. and Miller, J.J. “Development and fecundity of the manure worm, *Eisenia faetida* (Annelida:Lumbricidae), under laboratory conditions.” In: D.L. Dindal (ed.), “Soil Biology as Related to Land Use Practices.” Proc. 7th Internat. Soil Zool. Coll. of ISSS. EPA, Washington, DC., pp. 673–678 (1980).

(12) Weber, C.I., Horning, W.B., II, Klemm, D.J., Neiheisel, T.W., Lewis, P.A., Robinson, E.L., Menkedick, J.R., Kessler, F.A. “Short-term methods for estimating the chronic toxicity of effluents and surface waters to marine and freshwater organisms,” 2nd Edition. Environmental Monitoring Systems Laboratory, U.S. Environmental Protection Agency, Cincinnati, OH (600/4–87/028) (1988).